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## DIAGNOSTIC ELECTRON MICROSCOPY FOR AMELANOTIC MELANOMA: CORRELATION OF PATIENT BIOPSY, SOFT AGAR ASSAY, AND XENOGRAFT

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**SUMMARY.** In an attempt to diagnose a suspected amelanotic melanoma tumour, we examined a variety of tissue and cell samples from one patient at the ultrastructural level, which consisted of single cell suspensions of tumour cells with and without DOPA treatment, tumour cells after culture in agar with and without DOPA treatment, and single tumour cells heterotransplanted into a nude mouse. Premelanosomes were not observed in sections of the amelanotic tumour with routine electron microscopy. Osmophilic-dense bodies, suggestive of melanosomes, were noted in the single cells in suspension treated with DOPA and cells grown in agar without DOPA treatment. Definitive premelanosomes, with an identifiable striated matrix, were only observed in cells grown into colonies in agar and treated with DOPA. Positive L-DOPA reaction products were noted in the golgi complex, endoplasmic reticulum closely related to the golgi (GERL), and in vacuoles from cells grown in agar. As controls, Cloudman S91 53·1 melanoma cells were evaluated as single cells in suspension or as colonies after culture in agar, both with and without DOPA treatment. Premelanosomes were always observed in this established melanoma cell line while DOPA-treated cells contained positive L-DOPA reaction products. The overall findings identified the tumour as amelanotic melanoma and indicated that both DOPA treatment and culture in agar were needed for the demonstration of premelanosomes.

EARLY differential diagnosis of amelanotic melanoma from diffuse histiocytic lymphoma, large cell carcinoma of the lung, spindle cell sarcoma, and undifferentiated adenocarcinoma is important as these diseases have markedly different prognostic and therapeutic implications. Electron microscopic diagnosis of metastatic melanoma is based on the demonstration of cytoplasmic premelanosomes or melanosomes with a typical periodicity (Mackay and

Osborne, 1978). An abundance of melanosomes in the tumour simplifies this diagnosis. However, in amelanotic melanoma, diagnosis may be very difficult or impossible since positive identification of premelanosomes may be impossible as little or no melanin may be produced. In addition, the melanosomes may appear as irregular ovoid-dense bodies and must be distinguished from similar-appearing lysosomes. Identification may be further complicated by melanosomes which form dense aggregations in the tumour cytoplasm.

Recent attempts to find new ultrastructural criteria for the diagnosis of amelanotic melanoma in the absence of premelanosomes or demonstrable melanin have focused on parallel tubular arrays (Mackay and Silva, 1980). These parallel tubular arrays are clearly expressed in metastatic melanoma cells and in tissue culture (Mackay and Silva). Also, the ultrastructural DOPA reaction has contributed to improving the diagnosis of metastatic melanoma by highlighting the presence of melanin (Hunger, Paterson and Fairley, 1978; Anderson, Stevens and Moatamed, 1981). Other diagnostic techniques have included cytoenzymology (Rouge and Aubert, 1979), 5-S-cysteinyl-dopa assay (Rouge and Aubert), neurone specific enolase (Dhillon, Rode and Leatham, 1982), and heterotransplantation into nude mice (Aubert *et al.*, 1976). All these techniques represent an effort to identify methods to aid in the identification of amelanotic melanoma. Although these techniques have been useful, the initial diagnosis of amelanotic melanoma remains difficult.

A new specialised diagnostic technique for identifying melanoma is offered by combining the growth of melanoma cells in agar (Hamburger and Salmon, 1977; Meyskens *et al.*, 1981b) and the ultrastructural L-DOPA reaction. This specialised technique is also compatible with current melanoma chemosensitivity testing (Salmon *et al.*, 1978; Meyskens *et al.*, 1981a). Our study reports the observations of premelanosomes and melanin reaction products in the cells of a patient with amelanotic melanoma, a diagnosis made possible only by electron microscopic evaluation of cells grown in agar to colony size and subsequently treated with L-DOPA.

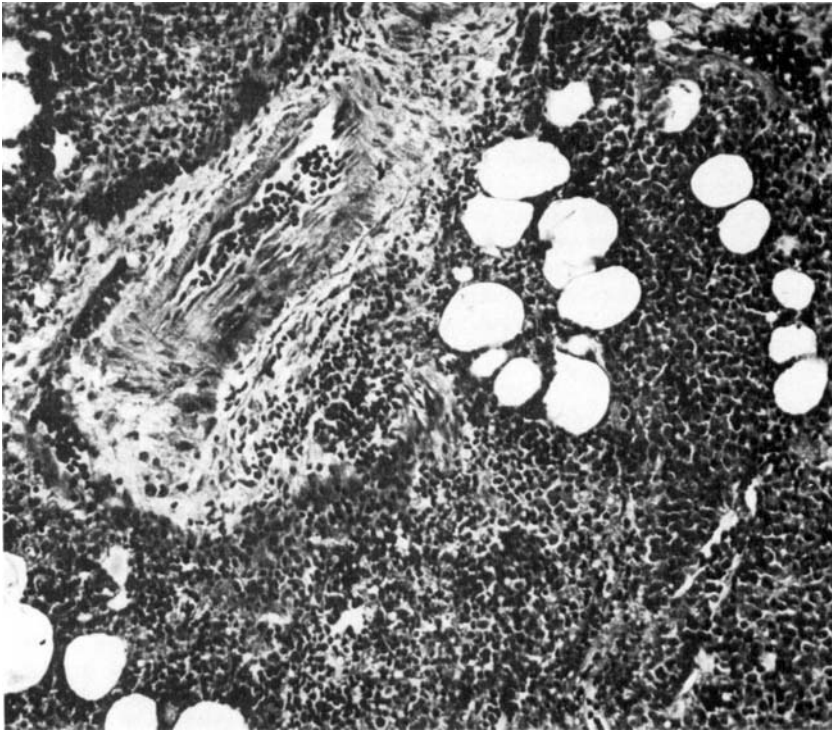
## MATERIALS AND METHODS

### *Case history*

The patient was a 33-yr-old white male who developed a level III malignant melanoma at age 25. The lesion was resected and no evidence of metastatic disease was detected. At age 32 a routine chest roentgenogram showed a solitary lung nodule. Further work-up indicated a left cervical node and biopsy showed the histology in the following figure. The patient was treated with experimental chemotherapy, but rapidly developed widespread metastatic disease and died 2 mth later.

### *Nude mouse xenograft*

A patient's tumour was excised in accord with a protocol approved by the University of Arizona Committee on Human Subjects. A single cell suspension was made by mechanical dissociation of the biopsy sample. From this suspension approximately 560,000 viable tumour cells, as determined by the trypan blue exclusion test, were injected subcutaneously into the left thigh of a 222-day-old female nude mouse. Six weeks later the mouse was sacrificed by cervical dislocation and the tumour was immediately excised and fixed (see Fixation).



Light Photomicrograph

This haematoxylin and eosin light photomicrograph from a lymph node section shows broad sheets of neoplastic cells surrounded by a small rim of adipose tissue. A few fibrovascular septa traverse the neoplasm and there are small islands of residual adipose tissue within the tumour mass. The neoplastic cells are large with large, slightly irregular, round to oval nuclei, demonstrating coarse chromatin clumping and prominent usually single nucleoli. Mitoses are quite frequent. There is a moderate amount of eosinophilic cytoplasm and the cell borders are indistinct. Focally, the cells appear to adhere together and deform each other. The malignant cells stain negatively with methyl green pyronin, melanin and PAS. The reticulin stain demonstrated reticulin fibres surrounding individual cells ( $\times 170$ ).

#### *Human melanoma clonogenic cell assay*

The culture system has previously been described in detail (Meyskens *et al.*, 1981b). Briefly, the patient's tumour biopsy was mechanically disrupted to form a single cell suspension. Cells were cryopreserved in Ham's F-10 medium, 10 per cent. fetal calf serum and 10 per cent. dimethylsulfoxide to a final concentration of 10 million cells per millilitre. Later, aliquots were thawed and processed for either growth in agar or single cell suspension studies. The cells were cultured in  $30 \times 10$  mm dishes (Falcon) with a 1.0 ml of underlayer containing 0.5 per cent. agar (Bacto) in Ham's F-10 medium supplemented with 10 per cent. heat-inactivated fetal bovine serum, penicillin (100  $\mu\text{g/ml}$ ) and streptomycin (100 units/ml). The plating layer was the same medium in 0.3 per cent. agar with freshly added animal-derived insulin (1.54 units/ml), glutamine (0.45  $\mu\text{g/ml}$ ), pyruvate (0.34  $\mu\text{g/ml}$ ) and mercaptoethanol (0.77 mM). Six dishes were plated with half a million cells each and incubated in a 5 per cent.  $\text{CO}_2$ , 95 per cent. air atmosphere at  $37^\circ\text{C}$  with constant high humidity. Colonies were fixed 21 days later. For the single cell suspension studies, two aliquots of thawed cells were fixed.

*Mouse melanoma clonogenic cell assay*

Cloudman's melanoma S91 (CCL) 53·1 were cultured in agar and in Falcon flasks for single cell suspension studies. Three agar plates of CCL melanoma were cultured in 30 × 10 mm dishes (Falcon) with a 1·0 ml of underlayer containing 0·5 per cent. agar (Bacto) in Ham's F-10 medium supplemented with 10 per cent. heat-inactivated horse serum, 2 per cent. heat-inactivated fetal calf serum, penicillin (100 µg/ml) and streptomycin (100 units/ml). The plating layer contained the same ingredients as the underlayer, but in 0·3 per cent. agar. After 10 days, the agar plates were fixed. For cell suspension studies, CCL cells were cultured in a Falcon flask containing Ham's F-10 medium supplemented with 10 per cent. heat-inactivated horse serum, 2 per cent. heat-inactivated fetal calf serum, penicillin (100 µg/ml), and streptomycin (100 units/ml). After four days a rubber policeman was used to remove the confluent monolayer of cells. The cells were gently centrifuged. After removing the supernatant, the cells were resuspended in fixative.

*Fixation and DOPA treatment*

There were nine different experimental groups: (1) nude mouse xenograft; (2) patient's tumour cells grown in agar with DOPA treatment; (3) patient's tumour cells grown in agar without DOPA treatment; (4) single cell suspension of patient's tumour with DOPA treatment; (5) single cell suspension of patient's tumour without DOPA treatment; (6) CCL cells grown in agar with DOPA treatment; (7) CCL cells grown in agar without DOPA treatment; (8) suspension of CCL melanoma cells with DOPA treatment; and (9) suspension of CCL melanoma cells without DOPA treatment.

The nude mouse xenograft was fixed in a modified Karnovsky fixative (1 per cent. paraformaldehyde, 1·25 per cent. glutaraldehyde, pH 7·3) and routinely processed for transmission electron microscopy. The remaining eight groups were processed as follows: all cells were fixed for 1 hr at room temperature in 3 per cent. glutaraldehyde in 0·1M phosphate buffer, pH 7·3. Fixation of the colonies in agar was accomplished by changing the fixative once during the hour. Cells were rinsed in 0·1M phosphate buffer, pH 7·3, for a total of 45 min. Half of each group was incubated for 4 hr in 5 mM L-DOPA in 0·1M phosphate buffer, pH 6·8, 37°C and the other half was incubated for 4 hr in 0·1M phosphate buffer, pH 6·8, 37°C. Cells were then kept at 4°C for 14 hr in their respective solutions, i.e., 5 mM L-DOPA in buffer or buffer only. Tissues were post-fixed in 1 per cent. OsO<sub>4</sub> in 0·1M phosphate buffer, pH 7·4, for 1 hr at room temperature, dehydrated in ethanol, followed by 100 per cent. propylene oxide, and placed overnight in a 50:50 mixture of propylene oxide-Spurr. Tissues were transferred to Spurr resin for 24 hr, and then finally embedded in fresh Spurr. Thin section were stained with uranyl acetate and lead citrate and examined in a Philips 300 transmission electron microscope.

## RESULTS

The nude mouse xenograft consists of cells with numerous mitochondria, golgi complexes, rough endoplasmic reticulum, and lipid-like inclusions (fig. 1). The nuclei are often convoluted with prominent nucleoli. Melanosomes, regardless of the developmental stage, are not observed in these preparations. The single cell suspension from the patient's tumour treated with DOPA contain DOPA-positive vacuoles and osmophilic structures suggestive, at low magnification, of melanosomes (fig. 2). However, these structures when viewed at higher magnification lack the characteristic striated matrix which would definitely classify them as melanosomes (fig. 3). These particular osmophilic structures are absent from the single cells in suspension which were not treated with DOPA. Definitive premelanosomes (fig. 4) and DOPA-positive golgi (fig.

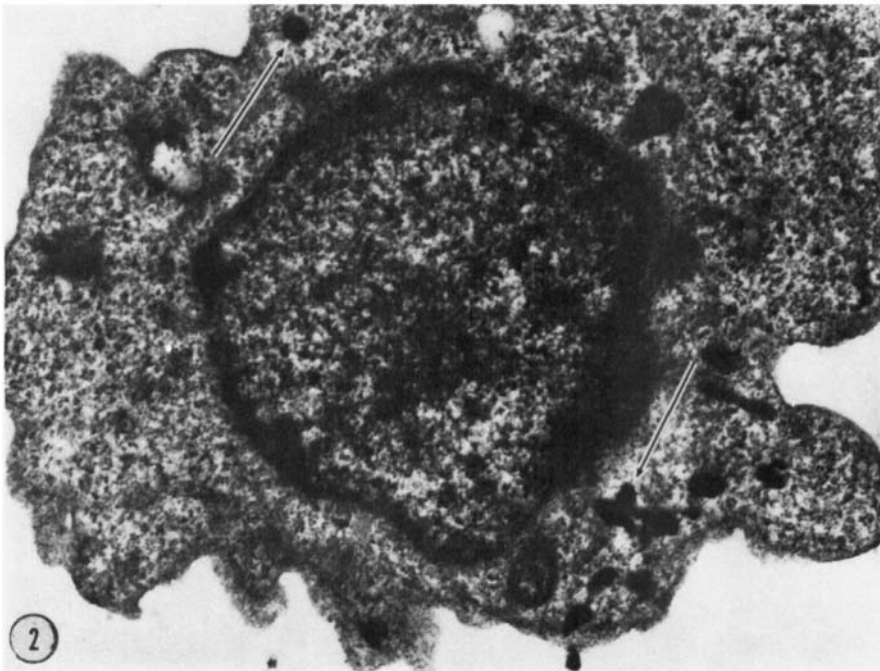
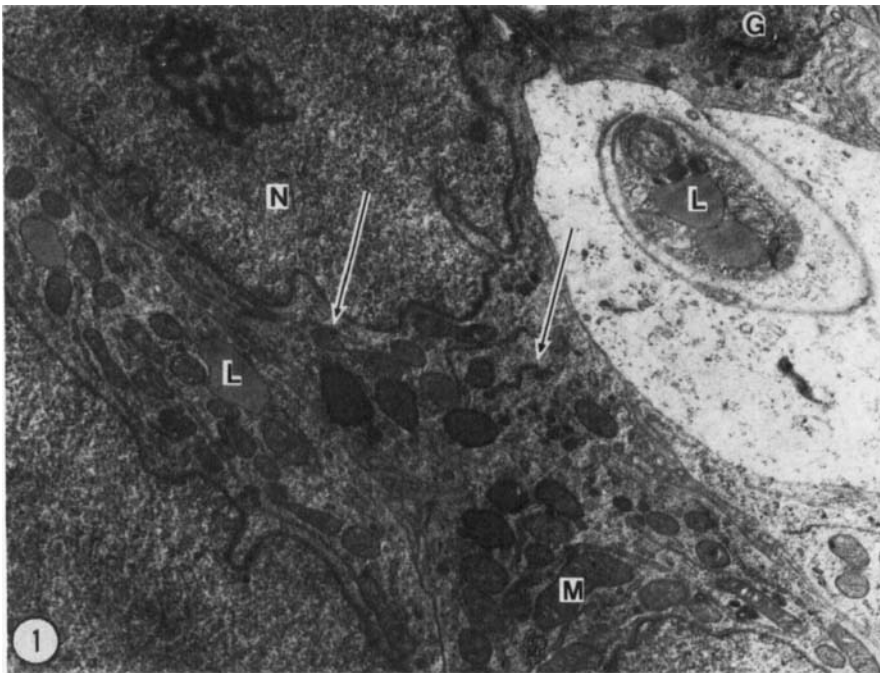


FIG. 1.—The nuclei (N) of the tumour cells are usually convoluted. The nucleolus is frequently visualised as thick strands. Mitochondria (M), Golgi complexes (G), rough endoplasmic reticulum (arrows), and lipid-like inclusions (L) appear normal. Melanosomes are not observed.  $\times 10,800$ .

FIG. 2.—Dense bodies (arrows) are noted in the cytoplasm.  $\times 23,400$ .

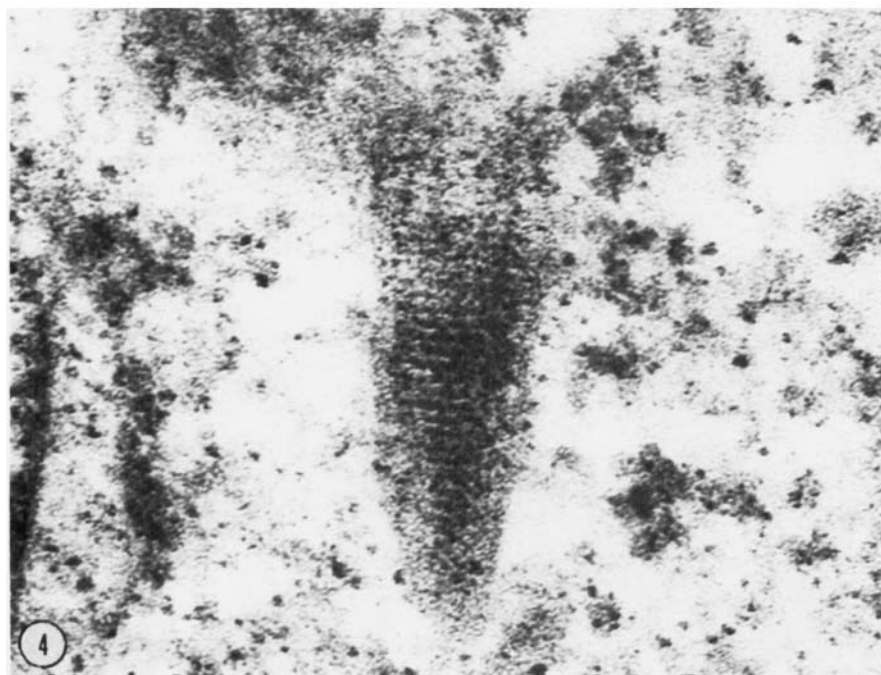
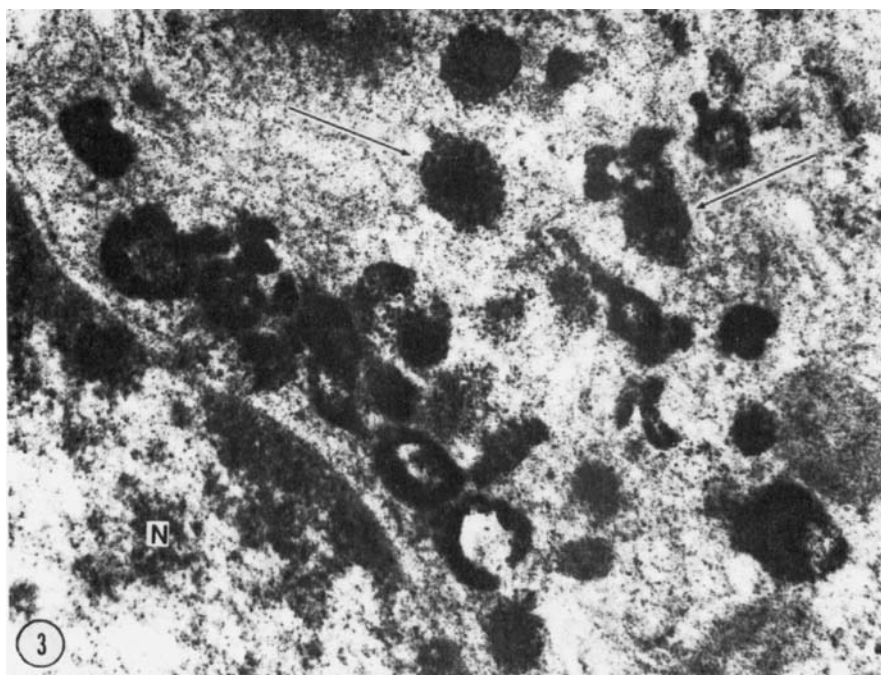


FIG. 3.—Higher magnification illustrates the granular composition of the dense bodies (arrows) in the cytoplasm. Note the absence of a striated matrix which would be indicative of premelanosomes. Nucleus, N.  $\times 103,500$ .

FIG. 4.—The striated matrix of a premelanosome is evident.  $\times 306,000$ .

5) are only observed in cells from colonies grown in agar and treated with DOPA. The DOPA reaction in the golgi complex is limited to a single cisterna, presumably associated with the maturing face of the golgi complex. Examination of cells grown in agar without DOPA treatment fail to reveal characteristic melanosomes, although osmophilic structures are noted in the cytoplasm (fig. 6). These structures, however, are not similar in morphology to those illustrated in fig. 3 since they are larger and generally have dense centres.

CCL cells express definitive premelanosomes in all situations (figs. 7–10). Treatment with DOPA of CCL melanoma cells in suspension, which served as a positive control, shows specific staining of vacuoles, golgi complexes, and related GERL (fig. 7), similar to that observed in the patient's cells cultured in agar and treated with DOPA. The golgi cisternae and related endoplasmic reticulum are not stained in the absence of DOPA treatment (fig. 10).

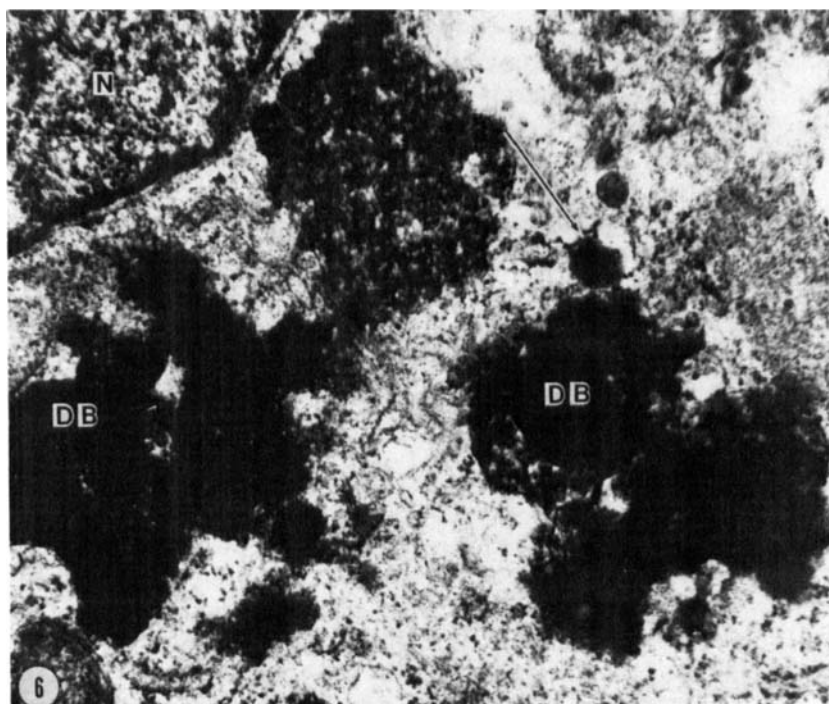
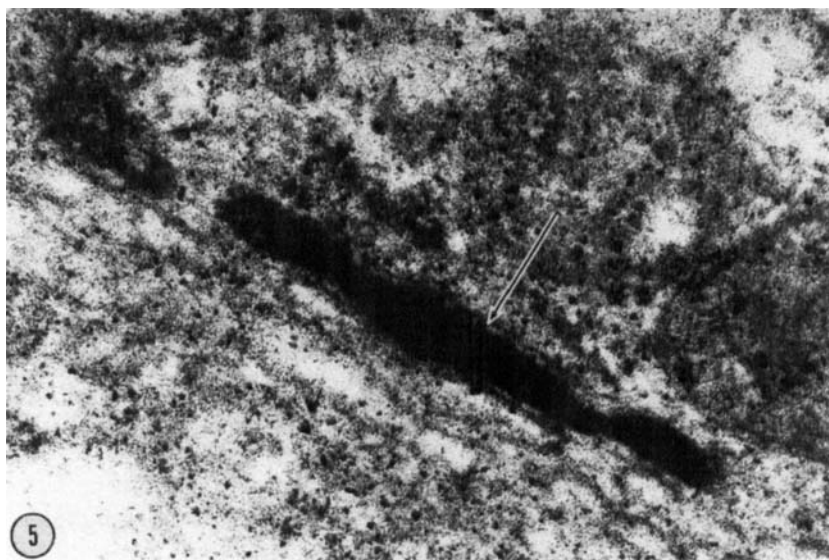
### DISCUSSION

Amelanotic melanoma is difficult to diagnose with current techniques. The histopathologic diagnosis is often complicated by the lack of melanosomes and pigment. Additionally, one-third of amelanotic melanomas present with an unknown site of the primary lesion while approximately 7 per cent. of pigmented melanomas have unknown primary sites (Shah, 1975).

Investigations utilising electron microscopy have morphologically described amelanotic and melanotic melanoma (Clark, Heggeler and Bretton, 1972; Hunter *et al.*, 1978; Mackay and Osborne, 1978; Mackay and Silva, 1980; Persky *et al.*, 1982). At present it is generally felt that the ultrastructural identification of premelanosomes is the definitive marker for melanoma. We undertook our investigation to locate and identify these characteristic structures in the cells from this patient. Transmission electron microscopy (TEM), the L-DOPA reaction and cell culture in agar were the diagnostic tools used in this study.

Anderson and coworkers (1981) recently demonstrated the applicability of combining TEM and the L-DOPA reaction in the diagnosis of amelanotic melanoma. The rationale behind the L-DOPA reaction was that since amelanotic cells lacked the enzyme tyrosinase they could not produce L-DOPA, and hence the end product melanin could not be synthesised. When the melanin precursor L-DOPA was introduced exogenously by incubation with the cells, melanin was synthesised. Since amelanotic melanoma has little or no melanin, the L-DOPA reaction amplified the melanin production and thus made it easier to identify premelanosomes by TEM and the protein machinery (organelles) involved in melanin production, specifically the golgi complex, the associated GERL, and the corresponding vacuoles. Not all melanoma cells treated with DOPA in our study demonstrated positive reaction products. This is not an unusual observation since cells are not expected to be involved in melanin production at all times. The marked differences in tyrosinase activities from cell to cell has been suggested as a possible drawback in any diagnostic procedure which might depend on DOPA uptake by malignant cells (Hunter *et al.*).





**FIG. 5.**—A single cisterna (arrow) of the golgi complex is DOPA-positive.  $\times 170,000$ .  
**FIG. 6.**—Both single (arrow) and aggregates of osmophilic dense bodies (DB) are present within the cell. Various degrees of osmophilia are observed. Nucleus, N.  $\times 57,800$ .

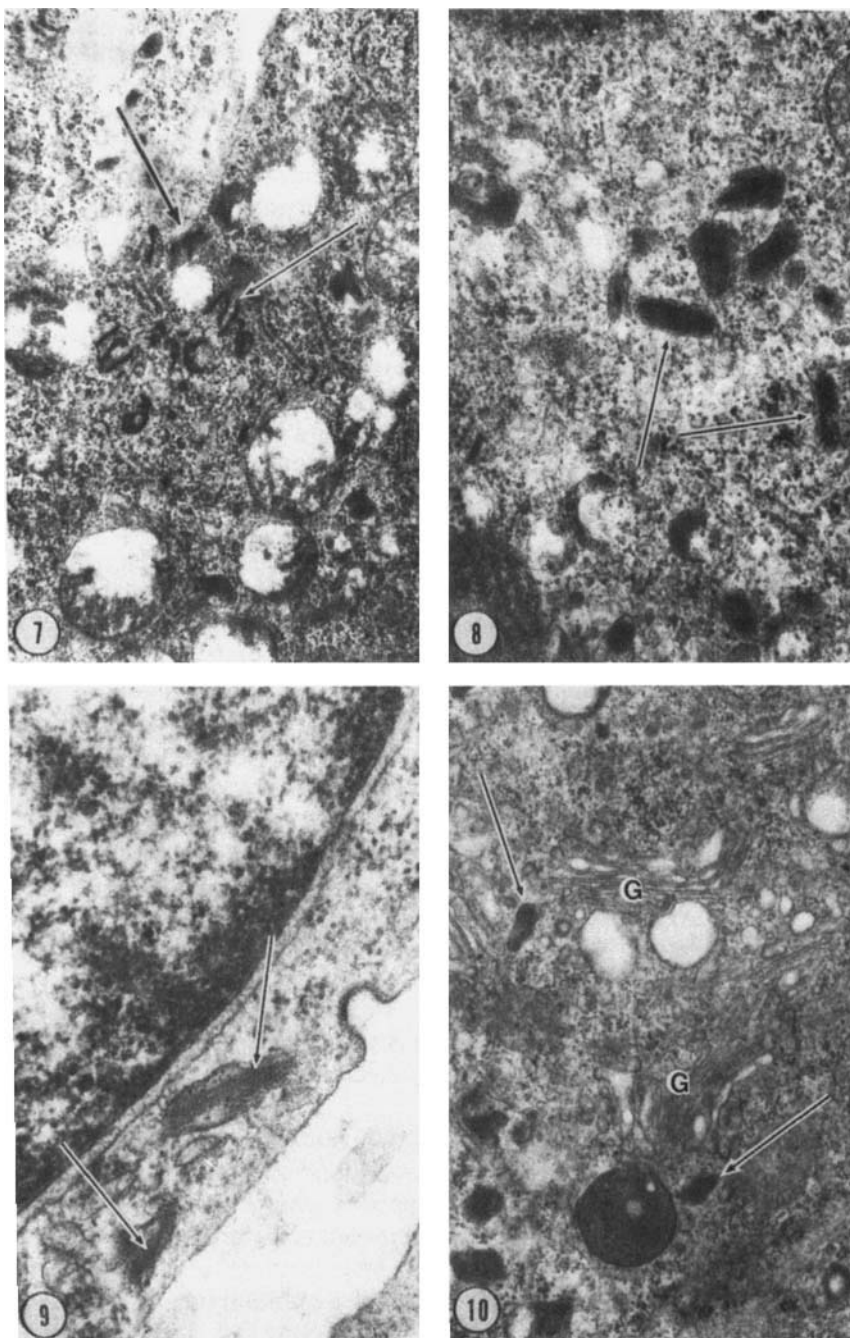


FIG. 7.—Single cell suspensions treated with DOPA demonstrate premelanosomes (large arrow) and DOPA-positive rough endoplasmic reticulum (small arrow).  $\times 42,240$ .

FIG. 8.—Premelanosomes (arrows) are identifiable in single cell suspensions without DOPA treatment.  $\times 50,160$ .

FIG. 9.—Similarly, premelanosomes (arrows) are recognised in cells grown in agar with DOPA treatment.  $\times 64,240$ .

FIG. 10.—CCL cells grown in agar, but without DOPA treatment, demonstrate premelanosomes (arrows) and golgi complexes (G).  $\times 23,760$ .

However, our study and others (Hunter *et al.*; Anderson *et al.*) have demonstrated a positive L-DOPA reaction in the golgi, the GERL, vesicles near the golgi and vacuolar melanosomes of melanotic and amelanotic cells. The fully characterised CCL line served as a convincing positive control for the DOPA reaction. Imperative to the L-DOPA reaction is that melanoma must be anticipated as a possibility in the diagnosis. Otherwise, to use the L-DOPA reaction as a routine screening test would be tedious, time-consuming, and impractical.

Cell culture in agar was a valuable method which assisted our clinical diagnosis of amelanotic melanoma as it permitted cells which previously did not demonstrate premelanosomes to now express premelanosomes. Although tissue culture is an important technique, it must be cautiously evaluated. Isolated cell lines of human melanoma have shown considerable variability in melanin content especially when considering the growth phase of cells in agar (Fitzpatrick *et al.*, 1979; Tveit *et al.*, 1980). Because of the paucity of melanin in the colonies grown in soft agar in our study, melanin variability, if present, was not detected or indeed just did not exist prior to L-DOPA treatment.

The heterotransplantation technique used in this study allowed multiple investigations that were not possible on small primary tumours. Recent studies have compared the ultrastructural characteristics, melanin content, isozyme pattern, and chromosome numbers of xenografts and found them to be similar to melanoma cell lines (Tveit and Pihl, 1981). Additionally, colonies cultured in soft agar from different cell lines are similar in size and morphology to their parent xenografts (Tveit and Pihl). There are, however, differences which have been reported between heterotransplanted tumour cells and tumour cells cultured in agar, such as growth rates *in vivo* and plating efficiencies in soft agar (Tveit and Pihl), tumorigenicity (Aubert, Rouge and Galindo, 1980) and pronounced changes in cell shape, melanosome morphology and 5-S-cysteinyl-dopa content (Aubert *et al.*, 1976). Although extrapolations from continuous in-vitro cell lines to in-vivo tumour cells may not always be valid, heterotransplantation will generally enhance locating premelanosomes as more material is available for analyses. In this study, however, heterotransplantation did not aid the diagnosis, since the nude mouse xenograft did not demonstrate premelanosomes.

Recent studies have reported specific markers for melanoma. The enzyme neuron specific enolase appears useful in identifying melanoma (Dhillon *et al.*, 1982) as does the visualisation of parallel tubular arrays at the EM level. Parallel tubular arrays, which have been reported to persist in tissue culture and also in later metastases (Mackay and Silva), were not present in the current case. Rouge and Aubert (1979) have suggested that a combination of the techniques of tissue culture, cytoenzymology, *in situ* TEM, and 5-S-cysteinyl-dopa assay offered valuable new approaches to the differential diagnosis of human malignant melanoma. Their report demonstrated 46 of 216 final diagnoses, as determined by one or more of the four techniques, differed from the initial histological diagnosis.

The ultrastructural identification of premelanosomes is currently the best criteria for diagnosing amelanotic melanoma. Only by combining the

techniques of the L-DOPA reaction and tissue culture could premelanosomes with an identifiable striated matrix be recognised. These parameters may be applied to ambiguous cases of malignancy for which amelanotic melanoma is suspected.

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